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Modeling Hemorrhagic Stroke and Vasospasm in the Rodent

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A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Abstract

In January 2010, I started work as an undergraduate research assistant at SUNY Upstate Medical University in Dr. Mary Lou Vallano's laboratory in the Department of Neuroscience and Physiology. My research project was part of a collaborative effort with the laboratory of Dr. Eric Deshaies, a vascular neurosurgeon at Upstate. The goal was to establish a rodent model of subarachnoid hemorrhage and delayed vasospasm, and to test possible protective strategies. We used an adult rat model, in which two injections of autologous blood were given in the cisterna magna region of the brain. Analysis was done using a combination of histology, immunohistochemistry, and molecular biology to examine vasospasm in the basilar artery, and changes in the surrounding neural tissues. Based on Dr. Deshaies' patient information and clinical work, we examined whether exercise preconditioning or the injection of stannous chloride would decrease the inflammatory response and the occurrence or intensity of vasospasm. Our results indicate that the model is working, and they point to the possible protective effect of stannous chloride.

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Background:

Subarachnoid hemorrhage (SAH) is bleeding into the subarachnoid space within the brain and is usually caused by a ruptured aneurysm. An aneurysms is a bulging or "ballooning" of an artery within the brain, which bursts to leak blood into the affected region As the red blood cells break down, oxyhemoglobin is released and contributes to oxidative stress responses in the environment. A number of symptoms are associated with this condition, including a severe headache, loss of consciousness, vision impairment, and delayed stroke due to ischemia (Welty et al., 1994; Dorsch and King, 1994). The initial aneurysm could be caused by many different factors, including a history of smoking, cocaine use, alcohol use, high cholesterol, hypertension and arteriosclerosis. It is estimated that one out of fifteen people will develop an aneurysm during their lifetime. However with this particular condition, there is no direct correlation to an age range, sex, heredity or ethnicity. My research aims to help develop a rodent model of SAH in our laboratory, provide information on the molecular changes in the vessel and surrounding brain tissue after SAH, and examine possible protective strategies to prevent cerebral vasospasm.

Stroke is the second leading cause of death in the world causing about 4.4 million deaths per year, and the third leading cause of death in the US. Only 10 percent of all stroke victims recover completely, 25 percent recover with minor impairments, and 40 percent have moderate to severe impairments requiring special care. Although SAH represents only 7 percent of all strokes,

they are the most deadly, with a fatality rate of ~25 percent (Dorsch and King, 1994; Lee et al., 2009). It has also been shown that about half the survivors have permanent disabilities. These high morbidities are believed to be due to a common complication that occurs days-weeks after the bleeding, which is cerebral vasospasm. Cerebral vasospasm is the narrowing or constriction of brain blood vessels, causing reduced blood flow to the brain. It is often associated with neuronal death or stroke due to oxygen and nutrient deprivation. Cerebral vasospasm develops in a majority of patients and up to 50% of affected patients have permanent damage (Welty et al., 1994; Dorsch and King, 1994). Clinically, SAH is confirmed with a computerized tomography scan (CT scan), which uses X-rays to create a three-dimensional image of the skull area. If this test is inconclusive, then the more invasive procedure of a lumbar puncture is used to identify specific proteins in the cerebrospinal fluid. Although it is possible to detect cerebral vasospasm in patients who have suffered a SAH, treatment options are limited.

Many different agents have been used for treatment including 'triple H therapy' corresponding to: hypervolemia (increasing blood volume); hemodilution (diluting plasma); and hypertension (elevating blood pressure). Many doctors also use strict bed rest. Neurosurgeons generally use calcium blockers to prevent vasospasm (Laskowitz and Kolls, 2010; Rabinstein et al., 2010). Currently though, despite decades of research, no therapies are convincingly effective in preventing cerebral vasospasm, or the neurological deficits following SAH. Experts agree that if we are to develop effective

therapies to prevent or reduce cerebral vasospasm, then we must understand the complicated cascade of molecular changes in the injured vessel that initiate and maintain it.

In the experimental portion of my capstone, I used Sprague-Dawley male rats as a model organism. Rats are useful organisms because they are cost-effective, and show similar physiological responses to SAH as humans, including delayed cerebral vasospasm (Tanaka et al., 2008; Lee et al., 2009). There are two widely used models to induce SAH in rats and mice. Endovascular perforation uses a fine wire to rupture the internal carotid artery. This method is more physiologically accurate to an actual SAH because it involves vessel damage, and release of arterial blood into the subarachnoid space under pressure. However, it is difficult to measure or limit the amount of blood that is released in the subarachnoid region of the brain. Because of this, the method has a fatality rate of ~50 percent causing more rats to be needed in each trial compared to other methods. Also, the extent of injury in surviving subjects is variable, which makes it difficult to evaluate protective agents. The method of choice in our laboratory is the double hemorrhage model (Lee et al., 2009). The double hemorrhage model involves two injections of arterial blood into the cisterna magna, given 24-48 hours apart. Delayed cerebral vasospasm is usually assessed between 2-7 days later. One of my initial goals was to help establish this model in our laboratory by: assisting with the surgeries, administering putative protective agents, perfusing the animals for sacrifice, preparing their tissue for subsequent assays, evaluating the basilar artery using

morphological, histological and immunological methods, extracting and processing mRNA for conversion to cDNA, and measuring differences in mRNAs using real-time RT-PCR and PCR arrays. I also trained subjects to exercise on a treadmill.

Vasospasm can be measured using angiography or histology. Since we did not have access to the expensive equipment needed for angiography, I used histology. I measured the internal and external perimeters of cross sections of the basilar artery. This was done on several sections of the artery after perfusion of the subject, sectioning of the vessel on a cryostat, and photographing the vessel cross sections prior to measurement. In many cases, I also stained the vessels with dyes or antibodies to permit improved imaging of various cells and layers.

To evaluate protective strategies, we focused primarily on the heme-oxygenase (HO-1) pathway. HO-1 is a heat shock protein, and the rate-limiting enzyme in heme catabolism. It is induced in several cell types in response to heat shock, stress, and heme. HO-1 may protect against oxidative stress by clearing heme from the subarachnoid space, and also through production of the antioxidant bilirubin. Consistent with this, HO-1 inhibition exacerbates cerebral vasospasm, and HO-1 gene therapy or induction with pharmacological agents attenuates it (Ono et al., 2002; Tanaka et al., 2008; Shimada et al., 2009) in rat models of SAH. Similarly, induction of other heat shock proteins (HSPs), including HSP-70/72, in response to SAH may contribute to protection (Nikaido et al. 2004; McLemore et al. 2005; Tanaka et al. 2008). We measured

changes in HSPs in control and SAH subjects using antibodies and real time RT-PCR. In some cases, we assessed broad changes in several HSP mRNAs in subjects receiving blood versus saline using PCR arrays specifying numerous HSP mRNAs. PCR arrays offer the opportunity to examine more than 80 mRNAs at once, as opposed to doing RT-PCR on one target at a time. This is important because it can provide a more complete picture of molecules that change in a coordinate fashion after SAH.

Stannous chloride ($SnCl_2$) has been shown to be protective in several injury models by inducing heme-oxygenase-1 (Deshaies et al., 2009; Perdrizet et al., 2002), but it has not been tested in a SAH model. One of my goals was to determine if injection of $SnCl_2$ provided protection against cerebral vasospasm in our model. Our working hypothesis was that injection of $SnCl_2$ after SAH would attenuate vasospasm, which was assessed histologically, and using RT-PCR or immunochemistry to measure molecules associated with protection or oxidative stress. Regarding molecular changes, we predicted decreases in certain oxidative stress molecules such as iNOS (injurious) and increases in certain heat shock and antioxidant defense molecules such as HO-1 and HSP-70/72 (protective).

In animal models, there is strong evidence that exercise preconditioning protects from global ischemic stroke (Ding et al. 2003; Ding et al. 2005). However, there is no information about the possible protective effect of exercise in SAH models. Another goal was to test the effects of treadmill exercise pre-conditioning on cerebral vasospasm after SAH. In histological

aspect, I looked for any differences between the basilar arteries of rats with exercise pre-conditioning followed by SAH versus those with SAH alone. I also examined the induction of immunoreactive iNOS in the vessels. Our working hypothesis was that two weeks of exercise pre-conditioning would attenuate vasospasm after SAH, compared to SAH subjects that did not exercise prior to injury. If this proved to be correct, it could change the way hypertensive patients are managed. Physicians could strongly recommend that their 'at-risk' patients exercise regularly giving the explanation that this could decrease the severity of a cerebral vasospasm in the event that they were to experience SAH. If patients were to follow this advice, it could save neurological function and even lives.

Method

In the following section, I describe the specific assays and protocols used in my research. They are divided into (a) the animal surgery, (b) the histology and (c) immunostaining of the vessel, (d) real-time RT-PCR, and (e) PCR arrays.

A. Surgery

This procedure was used to induce SAH and delayed vasospasm in our subjects. Over the course of my research, we injected different amounts of blood to determine what amount was optimal for causing vasospasm without causing significant mortality.

1. Rats were weighted and given an appropriate dose of ketamine/xylazine (0.07 mg/kg, ip), an anaesthetic rodent cocktail.
2. A catheter was inserted in the tail artery for blood removal (Figure 1).
3. After being anesthetized, the neck was shaved, animals were placed in the prone position, and a posterior cervical approach was used to make a midline suboccipital incision (Figure 2 shows exposed atlantooccipital membranes in a subject).
4. The cisterna magna was tapped with a 25-gauge needle and 0.2 mls of CSF was withdrawn and replaced with 0.2 ml of arterial blood.
5. The durotomy was glued closed with vetbond to prevent CSF leakage and loss of injected blood from the cisterna magna. The incision was closed with staples and the animal was inverted 20^{o} head down for 20

minutes to allow blood to congeal in the cisterns around the basilar artery (Figure 3).

6. The subject was sacrificed by perfusion between 90 minutes to 2 days later. The animals were anesthetized with Fatal Plus (0.2 ml/kg), subjected to transcardiac perfusion for histological and immunohistological examination. They are sacrificed by guillotine for RT-PCR and PCR array tissue preparation.

Figure 1: A catheter was used to obtain autologous blood from the tail artery.

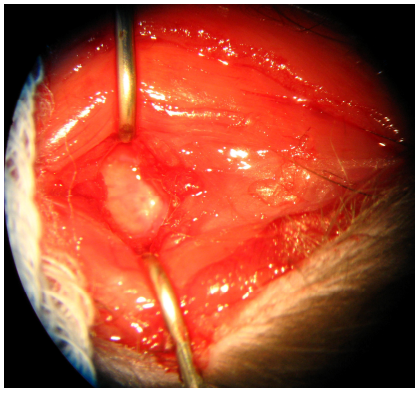


Figure 2: The cisterna magna region of the brain was exposed for injection.

The actual amount of the blood injected was varied from 0.15ml to 0.4mls in a series of trials to determine what would be optimal for producing a

reliable SAH and delayed vasospasm in the rats. Where indicated, we also used a sonicator to cause hemolysis of the blood before injection. This triggers a more intense and acute injury to the brain.

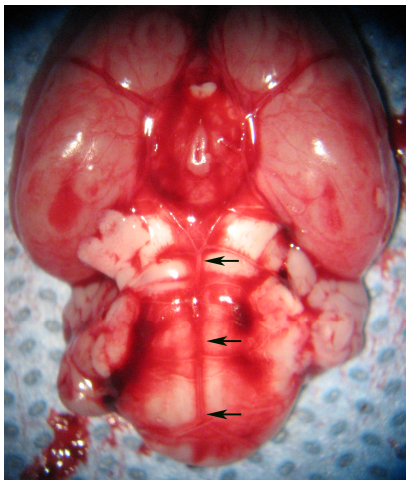


Figure 3: Ventral view of blood-injected brain showing blood pooling around the cisterns. Arrows depict basilar artery.

In a series of experiments focused on induction of HO-1 as a possible protective agent, $SnCl_2$ injection was examined. In these experiments, $SnCl_2$ (0.15 mg/kg) or saline was administered intraperitoneal 1-2 hrs before the second blood injection, and subjects were sacrificed 2 days later.

In another series of experiments, exercise preconditioning was examined for protective effects. Methodologically, I used a moderate conditioning protocol consisting of 2 weeks of treadmill running once daily at a rate of 15 M/min. We then tested four groups of rats—(1) the control group with just saline injection and no pre-conditioning exercise (no cerebral vasospasm is expected); (2) the blood-injected group with no exercise pre-

conditioning (to produce cerebral vasospasm); (3) the saline-injected group with two weeks of exercise pre-conditioning (a control for exercise alone—no cerebral vasospasm is expected); and (4) the blood-injected group with two weeks of exercise pre-conditioning (to reduce or prevent cerebral vasospasm).

B. Histology

The purpose of this method was to determine the actual characteristics of the basilar arteries within the brain after subarachnoid hemorrhage, and to assess whether delayed cerebral vasospasm was detectable. After sacrifice of the subjects by perfusion, we removed the relevant portion of brains, and prepared them for cryostat sectioning (12 uM sections) of the basilar artery in cross section, starting at the Anterior Inferior Cerebellar Artery (AICA) and ending at the Superior Cerebellar Artery (SCA). Using a Zeiss Image A1 with AXIO Vision 4.8 Software microscope, unstained sections were photographed, and then measured using NIH Image J Software. The basilar artery is located within the mid-ventral part of the brain, and carries oxygenated blood to the surrounding tissue such as the brain stem, cerebellum and temporal cortex. I hypothesized that when the animal has SAH followed by a cerebral vasospasm, the artery would become smaller in diameter and the inside walls would show evidence of corrugation. I performed two different measurements on multiple sections from each basilar artery. I measured the inside diameter of the vessel and the outside diameter of the vessel. Then these diameters were compared to those in other animals, and to those under other conditions.

C. Histochemical Staining

Several different stains were used to characterize both the molecular and physical characteristics of the basilar arteries. These included hematoxylin and eosin stain, fluorescent staining with iNOS, eNOS, and HSP-70/72 antibodies, and Masson's trichrome/Verhoeff stain.

1. Hematoxylin and Eosin Staining (H&E)

The hematoxylin and eosin was used to stain the basilar artery after SAH and delayed vasospasm. Hematoxylin stains cell nuclei blue, and eosin stains various organelles shades of pink and orange. There should be a significant difference in the morphology and staining patterns of the blood injected rats versus those injected with saline. This would be that the artery from the animal receiving blood would be because more constricted and the muscle layer more densely stained.

2. iNOS, eNOS, HSP-70/72 antibodies

Nitric oxide synthases are a family of enzymes that convert the substrate L-arginine to nitric oxide (NO) under different conditions. The eNOS isozyme corresponds to nitric oxide synthase in the endothelial layer of the vessel. Normally, it regulates vascular function by causing vasodilation. It was used to confirm the presence of the protein in the appropriate layer and also to assess possible changes between blood and saline groups.

The iNOS antibody stains for the inducible immune response that is activated in microglial and other cells in response to inflammation after SAH. This response be detrimental by interfering with eNOS function, and by producing excessive NO and generating oxidative stress. I hypothesized that there would be an increase in this protein in the adventitial (outer) layer of the basilar artery in animals injected with blood rather than saline.

HSP70/72 is a chaperone protein that is generated in the endothelial layer in response to several types of stress such as heat shock and exposure to specific chemicals. It helps protect cells by promoting the proper folding of misfolded proteins. I hypothesized that this protein would be increased in the endothelial layer of vessels from blood versus saline subjects in response to oxidative stress.

3. Masson's trichrome and Verhoeff stain

Masson's trichrome is a three-color stain that differentiates types of tissue based on color. Red is used to stain muscle fibers, blue to stain for collagen, and Verhoeff's stain to stain the elastic fibers black

D. Real-Time PCR

To assess molecular changes in mRNAs, I used real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assays, which involves the amplification of short segments of DNA from double-stranded DNA corresponding to specific mRNAs that are markers of cerebral vasospasm or

oxidative stress in surrounding tissues. This is a quantitative technique to reliably measure differences in such mRNAs. With these experiments, I looked at several molecules implicated in vasospasm. Special emphasis was given to HO-1, a potential protective enzyme that clears heme after a bleed (Suzuki et al., 1999; Shimada et al., 2009).

For real time RT-PCR

1. For extracting RNA, I used a Qiagen RNeasy Mini Kit.
2. RNA was converted to cDNA using Superscript III First-Strand Synthesis Supermix from Invitrogen.
3. To prepare PCR plates and detect amplicons, I used SYBR GreenER qPCR Supermix Universal from Invitrogen.

E. PCR Array

To gain a broader perspective about SAH-associated changes in HSPs, I purchased PCR arrays corresponding to genes associated with the heat shock family and stress markers. Each array contains 84 HSP genes and several housekeeping genes as controls. With my funds from SU Honors, I purchased the reagents needed to prepare the mRNA for analysis including the RT ^{2} First strand Kit from SA Bioscience. This "kit" is needed to convert the lysate (containing mRNA) to cDNA before doing PCR. It is essential in PCR experiments because it specializes in high-temperature capabilities as well as specific binding so that the results are accurate. The other reagents (e.g. SYBER Green) are needed to detect the amplified products.

Processing the tissue for the array proceeds as follows:

1. To extract tissue for RNA, I used the same kit as used for real-time RT-PCR, the Qiagen RNeasy Mini Kit.
2. To convert RNA to cDNA, I used RT First Strand Kit from SA Bioscience.
3. The array plate was SA Bioscience RT qPCR Master Mix.

Results:

In the following section, I describe the several specific examples of the results obtained through my research.

Using the double injection method, I assessed histological changes in the basilar artery of subjects injected with autologous blood (Figure 4) versus saline (Figure 5). As seen using a light microscope image of the vessel cross-section, there is significant constriction and thickening of the vessel walls after SAH. There is also corrugation of the basilar artery inner walls in the SAH subject.

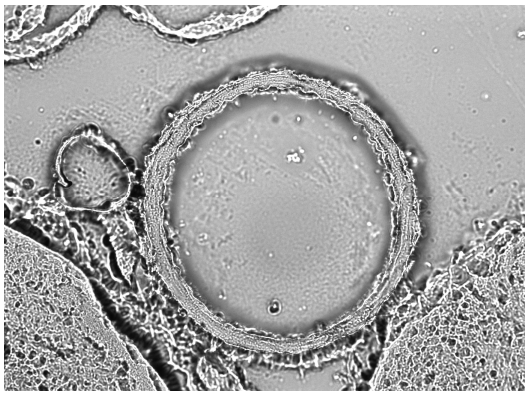


Figure 4: Basilar artery from blood-injected subject

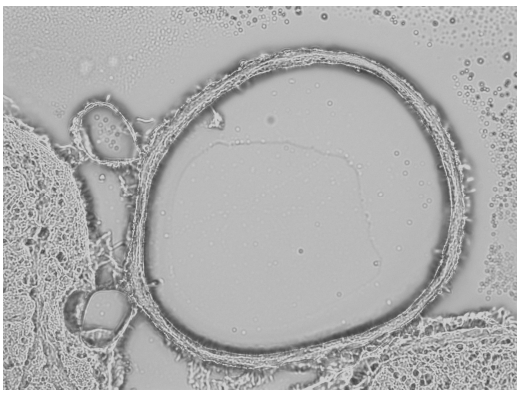
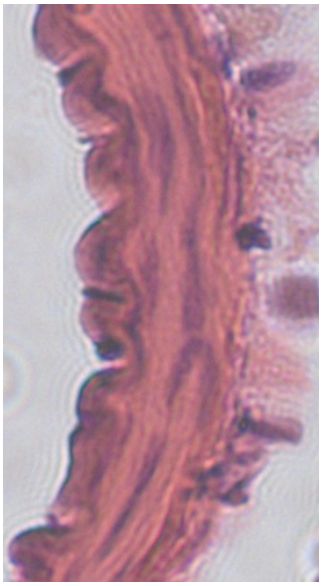


Figure 5: Basilar artery from saline-injected subject

H&E staining and higher magnification images were used to distinguish between the different layers in the basilar artery (Figures 6 & 7). The thickened walls and corrugated intimal layer are apparent in the subject that received blood injections, compared to the saline control. In the control subject, the layers of the vessel are distinct and have a banding pattern. These layers are mixed and not as uniform in the SAH subject.



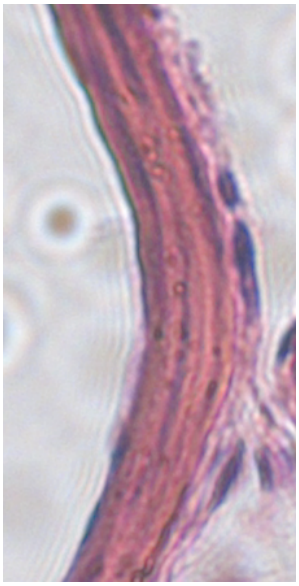


Figure 6 (left panel): H&E stain of basilar artery from blood-injected subject.
Figure 7 (right panel): H&E stain of Basilar Artery from saline control subject.

Although I frequently observed substantial vasospasm in blood-injected versus saline-injected subjects, this was not always the case. In an effort to find a more reliable marker of injury, we stained vessels with antibodies against the inflammatory protein, iNOS. As shown, iNOS protein is induced in the outer adventitial layer of basilar arteries from blood-injected subjects. It is not detected in arteries from saline-injected subjects (Figure 8).

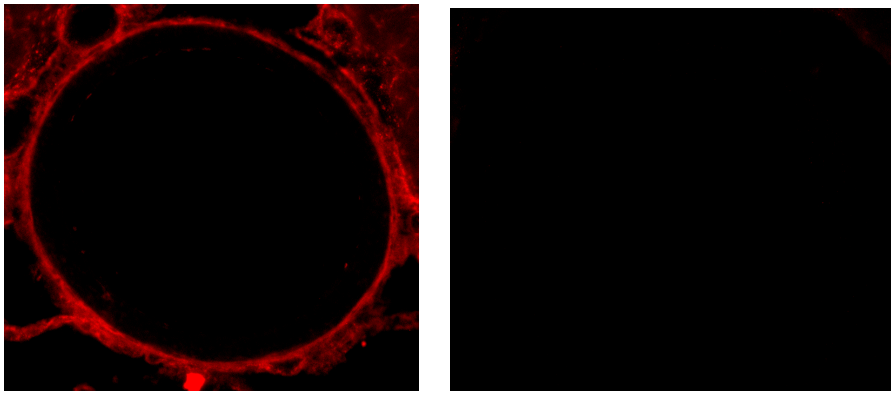


Figure 8: iNOS immunoreactivity: left panel is the basilar artery from a blood-injected subject; right panel is the basilar artery from a saline-injected subject.

There are three primary layers within the artery walls. The tunica intima is a single layer of epithelial cells at the innermost region of the artery. The tunica media contains smooth muscle cells and elastic tissue. The outermost tunica adventitia contains fibroblasts, collagens and elastic tissue. Figure 9 is a higher magnification of a blood-injected subject depicting these layers. Note that nuclear staining is blue and iNOS staining is red.

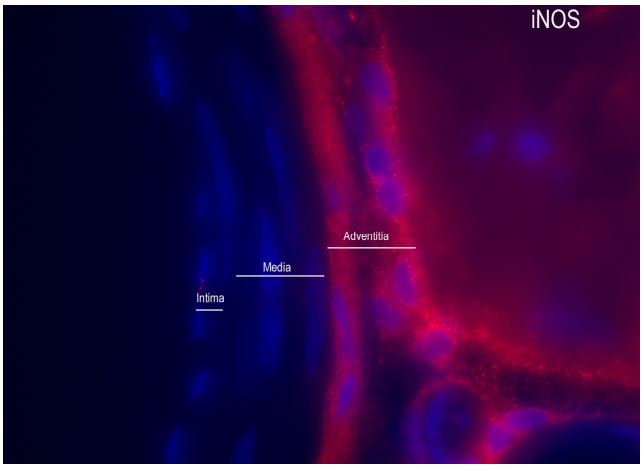


Figure 9: iNOS staining (red) in the adventitial layer of a blood-injected subject. Cell nuclei are shown in blue.

The enzyme eNOS normally functions to generate NO and cause vessel dilation as needed to maintain an adequate blood supply to the tissues. After SAH, several processes that are still being defined compromise eNOS function, contributing to delayed vasospasm. As predicted, eNOS immunoreactivity (Figure 10) is localized to the endothelial layer of the basilar artery. We did not observe consistent changes in eNOS immunoreactivity in blood versus saline-injected subjects.

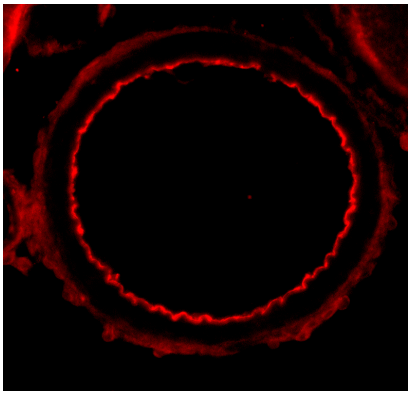


Figure 10: eNOS Staining of basilar artery from a blood-injected subject.

A possible protective response after injury is increased expression of HSPs. For example, Tanaka and colleagues (2008) showed that both HO-1 and HSP-70/72 are increased in the basilar artery of a mouse model after SAH. When these proteins were inhibited using antisense oligonucleotides, cerebral vasospasm was aggravated. On the other hand, use of drugs that enhanced their expression reduced vasospasm. Based on this and other related work, we examined whether these proteins were increased in the basilar artery after SAH in our rat model. Indeed, the heat shock protein HSP-70/72 (Figure 11) is

induced in the intimal layer in subjects that received blood compared to the saline controls.

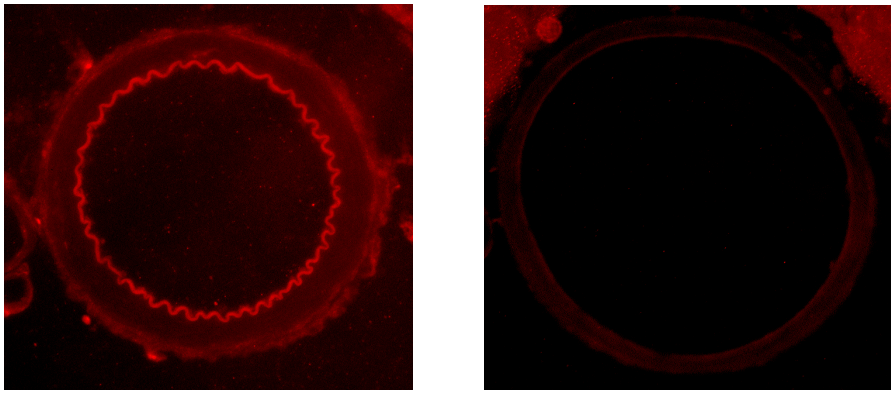


Figure 11: HSP 70/72 Staining of Basilar Artery: left is the blood-injected subject; right is the saline-injected subject.

We also attempted to detect induction of HO-1 protein in the basilar artery of blood-injected versus saline-injected subjects. However, we were unable to detect any staining at all with the HO-1 antibodies that were used. As an alternative, we used real-time RT-PCR to examine induction of HO-1 mRNA in neighboring tissues. Figure 12 is an amplification curve showing a 2-3-fold increase in amplicon corresponding to HO-1 mRNA in two different tissues from the blood-injected rats compared to the saline injected rats. This is reflected by a leftward shift in the amplification curve for the blood-injected subjects, compared to the saline-injected subjects. This increase is expected because the blood-injected rats are exposed to stress while they are enduring SAH, and HO-1 induction compensates to clear the heme.

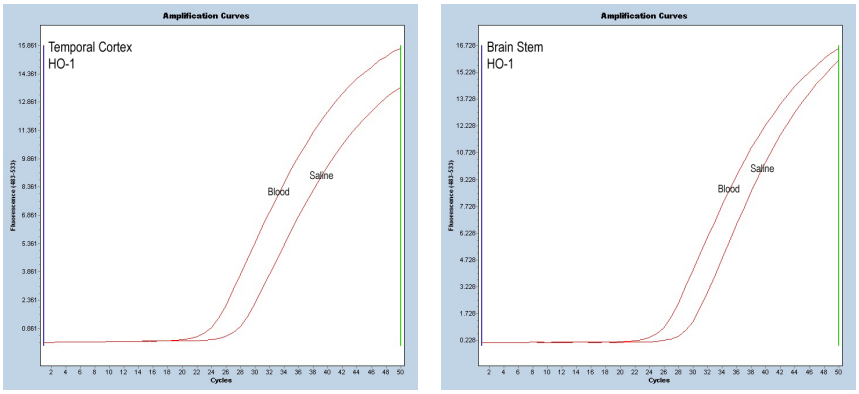


Figure 12: Amplification curve showing increased HO-1 mRNA in temporal cortex (left) and brain stem (right) in subjects injected with blood versus saline.

As discussed previously, we were interested in testing the hypothesis that injection of $SnCl_2$ would be protective against cerebral vasospasm, perhaps by boosting the induction of HO-1 (and possibly other protective HSPs) after SAH. This is based on evidence in other models of injury including an ischemia-reperfusion model used by our collaborator (Deshaies et al., 2009). Initially, we documented that injection of $SnCl_2$ (0.15mg/kg) induces HO-1 mRNA in our rat model. To test this, subjects were injected with $SnCl_2$ or saline, and then sacrificed 18 hours later when HO-1 mRNA was expected to be significantly induced. Brain stem was removed and RNA extracted and processed using real-time RT-PCR. As shown in the amplification curve, HO-1 mRNA was enhanced ~6-8-fold in the two subjects that received stannous chloride versus saline subjects (Figure 13). This is reflected by a leftward shift in the amplification curve.

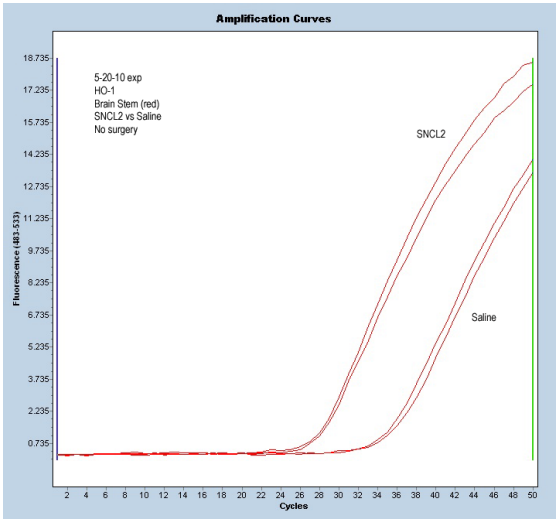


Figure 13: Amplification curve showing increased HO-1 mRNA in brain stem in subjects injected with $SnCl_2$ and extracted 18 hours later.

Next, we examined the effects of injection of $SnCl_2$ as a protective strategy in subjects exposed to SAH, compared to saline control subjects, and subjects receiving only blood injection without $SnCl_2$. We chose to inject $SnCl_2$ after the 1st blood injection, and just prior to the 2nd blood injection in our double injection model of SAH. Figure 14 shows examples photographs of the basilar arteries from a saline control, a blood alone, and a blood plus $SnCl_2$ that showed morphological evidence of protection. Below the images are internal and external diameter measurements of several cross-sections of the vessels, and their averages. As shown, the blood-only subject demonstrated obvious vasospasm, reflected by a thickened medial layer and corrugation of the intimal layer. In contrast, the saline control, and blood plus $SnCl_2$ subjects showed normal-looking vessels. We observed this apparent protective effect in half (3 of 6) of our subjects. Although we would like to measure changes in

several HSPs in the basilar arteries of subjects under different conditions, we were unable to obtain adequate amounts of mRNA or protein for testing in these small vessels.

Blood **$SnCl_2$** **Saline**

0.04	0.052	0.073	0.084	0.096	0.112
0.044	0.056	0.103	0.117	0.087	0.102
0.043	0.058	0.092	0.106	0.094	0.107
0.045	0.058	0.103	0.118	0.082	0.097
0.042	0.057	0.101	0.114	0.088	0.104
0.042	0.055	0.099	0.113	0.081	0.095
0.042	0.058	0.101	0.115	0.077	0.091
0.044	0.056	0.094	0.109	0.091	0.108
0.046	0.057	0.098	0.112	0.085	0.101
0.043	0.057	0.067	0.078	0.093	0.108
0.04	0.057	0.104	0.118	0.099	0.116
0.041	0.061	0.099	0.126	0.093	0.112
0.042	0.056	0.111	0.084	0.096	0.117
0.042	0.056	0.074	0.086	0.102	0.099
0.042	0.055	0.075	0.084	0.083	0.099
0.041	0.058	0.064	0.077	0.085	0.103
0.039	0.056	0.073	0.092	0.088	0.107
0.043	0.058	0.067	0.115	0.094	0.105
0.04	0.055	0.079	0.115	0.09	0.117
0.04	0.055	0.101	0.077	0.102	0.108
0.042158	**0.05655**	**0.088778**	**0.101941**	**0.089412**	**0.104611**
	74.40%		**87.20%**		**85.70%**

Figure 14: Comparisons of vessel cross-sections in subjects injected with blood (left), blood + $SnCl_2$ (middle), and saline (right). Actual measurements made using NIH Image J are shown below (internal diameters on left, external diameters on right).

I made similar measurements of basilar artery cross-sections in subjects injected with saline versus blood following 2 weeks of moderate exercise preconditioning versus no exercise. However, we were not able to detect significant morphological or immunochemical differences between the exercised and non-exercised groups (data not shown).

We were also interested in testing acute changes in HSPs following a more intense form of injury that was expected to cause substantial cell death in the tissues surrounding the basilar arteries. This type of injury may more closely mimic the most severe cases of SAH. To test this, whole blood was sonicated to disrupt, or hemolyze, the red blood cells prior to a single injection. After sacrifice, RNA was extracted from the temporal cortex and PCR arrays for several HSPs were used to compare subjects injected with 0.4 ml hemolyzed blood or saline, and sacrificed at 90 minutes or 2 days. I processed PCR arrays using tissue from 4 animals (saline versus blood-injected; sacrificed at 90 minutes or 2 days), and another person from the laboratory processed separate arrays using tissue from 4 different animals exposed to the same conditions. When then compared data for reproducible changes. A total of 6 mRNAs were significantly decreased in our blood-injected groups, compared to our saline control groups that were sacrificed after 90 minutes (Table 1). The decreases were observed in mRNAs encoding activating transcription factor 6, two HSP 40 family members, a HSP-70/72 family member, and two prefoldin subunits. It will be necessary to develop primers for each of these mRNAs and verify these data independently using real-time

RT-PCR. No common changes between our arrays were observed in blood-injected versus saline-injected subjects sacrificed at 2 days. Also, no mRNAs were commonly increased at either time point of sacrifice.

Table 1: Rat Heat Shock Protein PCR Array 90 minute time point

AtF6	**Activating Transcription Factor 6**	**↓ 2.2 fold**
Dnaja4	**HSP 40 family**	**↓ 2.4 fold**
Dnajb5	**HSP 40 family**	**↓ 3.2 fold**
Hspa2	**HSP70-72 family**	**↓ 4.0 fold**
Pfdn1	**Prefoldin subunit 1**	**↓ 2.6 fold**
Pfdn5	**Prefoldin subunit 5**	**↓ 3.1 fold**

Discussion

Cerebral vasospasms are widely thought to cause the most complicated and deadly outcomes after subarachnoid hemorrhage. Specifically, reduced arterial diameter interferes with blood flow, depriving the brain of oxygen and resulting in a secondary stroke. Up to 75 percent of patients with subarachnoid hemorrhages develop vasospasm, and 12 percent of these people have severe, permanent damage. However, due to the delayed nature of the vasospasm, there is a window of opportunity to intervene prior to ischemic injury to neurons (Dorsch and King, 1994; Laskowitz and Kolls, 2010; Rabinstein et al., 2010). If reliable, reproducible therapies were developed, many patients could avoid long hospital stays and thousands of dollars in health care fees.

We explored two theories of protection in a rat model of SAH. The first was that $SnCl_2$ injection would decrease the severity of vasospasm by inducing heat shock proteins, in particular HO-1. The second was that moderate exercise on a treadmill for 2 weeks would be protective against vasospasm severity. It is known that exercise preconditioning reduces neurofunctional damage in global ischemic models of stroke (Ding et al., 2003; Ding et al., 2005), but this strategy had not been tested in hemorrhagic stroke models. Thus, the goals of our research were to explore the benefits of these interventions as possible therapies for this deadly condition, after first developing and characterizing the double blood injection model of SAH in the rat.

Rats are useful model organisms because they are inexpensive, and have been widely employed to study SAH, delayed vasospasm, and neuronal

damage (Tanaka et al., 2008; Lee et al., 2009). Although it would be ideal to work with primates because they are the most closely related to humans, these subjects are quite expensive and many laboratories do not have the facilities to house them. Of the two best studied models of SAH and vasospasm in the rat, we chose to inject arterial blood into the cisterna magna, instead of perforating the internal carotid artery for two reasons: the mortality rate is much lower, and one has better control over the amount of blood or blood products being injected into the experimental subject (Lee et al., 2009).

I devoted substantial effort towards the histological analysis of basilar artery cross-sections in subjects injected with blood, saline, or nothing. Although I observed several examples of decreased vessel cross-section in subjects injected with blood versus control subjects, this was not a consistent effect. One potential problem with the histological analysis of vessel cross-sections is that all portions of the artery are not necessarily in vasospasm. Another potential problem is found regarding individual differences in the responses of different subjects. Finally, the timing of maximal vasospasm and resolution of vasospasm appears to differ among subjects (Tanaka et al., 2008; Lee et al., 2009). Thus, to assess vessel injury after SAH independent of obvious vessel constriction, we used antibodies against the inflammatory marker iNOS.

Although the pathogenesis of cerebral vasospasm is complex and multifaceted, there is good evidence that hemoglobin-induced oxidative stress is a key contributor. Consistent with this, we observed an increased expression

of immunoreactive iNOS in the adventitial layer of the basilar artery in subjects receiving blood, in contrast to saline or no injection controls. This protein is induced in microglia and other cells that are recruited to the adventitial layer of the vessel in response to the inflammatory environment. It generates NO and oxidative stress that will injure cells and tissues if it is not resolved in a timely manner. In contrast, specific HSPs are thought to be induced after SAH as a means of handling the injury and stress (Nikaido et al. 2004; McLemore et al. 2005; Tanaka et al. 2008). For example, injection of HO-1 antisense oligonucleotides interferes with HO-1 mRNA induction and worsens vasospasm in the rat model (Suzuki et al. 1999), whereas boosting HO-1 using pharmacological agents or gene therapy is protective (Ono et al., 2002; Shimada et al. 2009). Other HSPs, such as HSP-70/72 and HSP-40 act as chaperones to prevent the accumulation of damaged, unfolded proteins. As predicted, we observed induction of immunoreactive HSP-70/72 in the intimal layer of the basilar artery in rats receiving blood, compared to saline controls. Although our HO-1 antibody did not show detectable staining of the basilar artery under any conditions, we were able to demonstrate significant induction of HO-1 mRNA after blood injection or $SnCl_2$ injection in neighboring tissues using a real-time RT-PCR assay.

We also explored an injury model in which acute tissue damage was expected. This modified paradigm involved injection of a large volume of hemolyzed blood to produce a more acute and intense injury. We observed greater mortality in our subjects (~40%), in contrast to injections of smaller

volumes of autologous whole blood. In this series of experiments, I used PCR arrays of HSPs to assess evidence of tissue damage that could not be overcome or compensated by induction of HSPs. We were able to reproducibly detect decreases in 6 mRNAs in the 90 minute SAH sacrifice group, compared to saline controls. These mRNAs encoded activating transcription factor 6, two HSP 40 family members, a HSP-70/72 family member, and two prefoldin subunits. There is little information known about prefoldins, but the decreases in HSP 40 and 70/72 family members are interesting. These mRNAs encode chaperones that work together to prevent the accumulation of misfolded proteins and promote cell survival under stressful conditions. The observed decreases may reflect cell death in a system that is overwhelmed by the intensity of the injury.

Stannous chloride has been documented to induce heat-shock proteins and to afford protection in several injury models, though it has not been previously tested in a SAH model. For example, it decreases spinal cord injury after aortic surgery (Perdrizet et al 2002), and decreases neuronal injury in an acute-global ischemia/reperfusion model (Deshaies et al 2009). We obtained promising preliminary data that $SnCl_2$ reduces oxidative stress and protects against vasospasm in the rat model. Initially, we found that injection of $SnCl_2$ significantly induces HO-1 mRNA in our rat model based on real-time RT-PCR analysis. Based on histology and iNOS staining of the basilar artery, our data showed that 3 of 6 subjects showed reduced evidence of vasospasm when injected with blood and $SnCl_2$, in contrast to saline controls. Future studies in

the rat model will focus on optimizing the time of injection and dosage. Also, the number of test subjects needs to be increased to permit statistical analysis.

Subarachnoid hemorrhages are intensely complex and heterogeneous injuries. It is likely that successful treatment will require a combination of therapies applied at different times after injury. Although we did not observe evidence of protection with exercise pre-conditioning, further development of the paradigm, perhaps using molecular markers of exercise intensity, may prove successful. Our other strategy suggests that the use of $SnCl_2$, by inducing HSPs and, possibly, by reducing iNOS and oxidative stress, appears promising. These are exciting results that may ultimately pave the way for clinical trials in patients, since $SnCl_2$ is FDA-approved and safe as a component in contrast agents for use in humans.

References:

Dorsch NWC, King MT: A review of Cerebral Vasospasm in Aneurysmal Subarachnoid Hemorrhage: I, Incidence and effects J Clin Neuroscience 1:19-12, 1994.

Laskowitz, D, Kolls, B; Neuroprotection in Subarachnoid Hemorrhage; Stroke 2010;41;S79-S84, 2010.

McLemore EC, Tessier DJ, Thresher J, Komalavilas P, Brophy CM (2005). Role of the small heat shock protiens in regulating vascular smooth muscle tone. J AM Coll Surg 201: 30-36.

Nikaido H, Tsunoda H, Nishimura Y, Kirino T and Tanaka T (2004). Potential role for heat shock protein 72 in antagonizing cerebral vasospasm after rat subarachnoid hemorrhage. Circulation 110: 1839-1846.

Rabinstein, A, Lanzino, G, Wijdicks, E; Multidisciplinary management and emerging therapeutic strategies in aneurysmal subarachnoid hemorrhage; Lancet 9: 504-519, 2010.

Tanaka T, Oka T, Shimada Y, Umemoto N, Kuroyanagi J, Sakamoto C, Zang L,Wang Z and Nishimura Y (2008). Pharmacogenomics of cardiovascular pharmacology: pharmacogenomic network of cardiovascular disease models. J Pharmacol Sci 107: 8-14.

J-Y Lee et al. (2009) Comparison of experimental rat models of early brain injury after subarachnoid hemorrhage. Neurosurgery 65 (#2): 331-343.

H Suzuki et al. (1999) Heme oxygenase-1 gene induction as an intrinsic regulation against delayed cerebral vasospasm in rats. J. Clin Invest: 104: 59-66.

Y. Shimada et al. (2009) Synergistic induction of heme-oxygenase-1 by nicaraven after subarachnoid hemorrhage to prevent delayed cerebral vasospasm. Eur. J. Pharmacol. 620: 16-20.

Welty TE. (1994) Prior Calcium-Channel Antagonist Use and Ischemic Stroke Outcomes. Ann Pharmacother. 28(5): 671.

Ono S, Komuro T, Macdonald RL (2002) Heme oxygenase-1 gene therapy for prevention of vasospasm in rats. J Neurosurg. 96(6):1094-102.

Deshaies EM, Boulos AS, Drazin D and Popp AJ (2009). Evidence-based pharmacotherapy for cerebral vasospasm. Neurol Res 31: 615-620.

Deshaies EM, Boulos AS and Popp AJ (2009). Peri-operative medical management of cerebral vasospasm. Neurol Res 31: 644-650.

Perdrizet GA, Lena CJ, Shapiro DS and Rewinski MJ (2002). Preoperative stress conditioning prevents paralysis after experimental aortic surgery: increased heat shock protein content is associated with ischemic tolerance of the spinal cord. J Thorac Cardiovasc Surg 124: 162-170.

Ding Y, Li J, Clark J, Diaz FG and Rafols JA (2003). Synaptic plasticity in thalamic nuclei enhanced by motor skill training in rat with transient middle cerebral artery occlusion. Neurol Res 25: 189-194.

Ding YH, Young CN, Luan X, Li J, Rafols JA, Clark JC, McAllister JP, 2nd and Ding Y (2005). Exercise preconditioning ameliorates inflammatory injury in ischemic rats during reperfusion. Acta Neuropathol 109: 237-246.

Summary

According to a study at the University of Medicine and Dentistry of New Jersey, stroke is the second leading cause of death in the world, causing about 4.4 million deaths per year, and the third leading cause of death in the U.S. The study also finds that only 10 percent of all stroke victims recover completely, 25 percent of stroke victims recover with minor impairments and 40 percent of victims have moderate to severe impairments requiring special care.

Stroke is any interruption of blood flow within the brain that can cause damage (Welty et al., 1994; Dorsch and King, 1994). Put another way, it is a heart attack within the brain. There are two types of strokes—ischemic and hemorrhagic stroke (National Stroke Association). Ischemic stroke occurs because a clot (thrombosis), or fat deposit, causes a blockage within a vessel in the brain (National Stroke Association). It can also occur when part of a blood clot elsewhere in the body t breaks off and travels up to the brain (National Stroke Association). Hemorrhagic stroke is caused by an aneurysm, or a weakened blood vessel, that breaks open, leaking blood into the brain or space around it (National Stroke Association). This causes uncontrollable bleeding within the brain, resulting in a number of problems and secondary conditions.

Symptoms of a stroke vary based on the individual victim. They can cause complete numbness of one side of the body, severe headaches, dizziness and vision impairment, trouble speaking, loss of understanding and sudden lack of coordination (National Stroke Association). With any of these warning

signs, it is important to get to a hospital as soon as possible because minutes can make a difference in treatment and recovery (National Stroke Association). There are many different effects of stroke, depending on the location where the stroke occurred. If it occurs on the right brain, it can cause paralysis of the left side of the body, vision problems and memory loss (National Stroke Association). If the stroke occurs on the right, it can cause paralysis of the left side of the body, speech problems and memory loss (National Stroke Association).

Hemorrhagic stroke has been the target of my research at SUNY Upstate for the one and a half years. Specifically, we are looking at subarachnoid hemorrhages, which is bleeding in the subarachnoid space. This space is between the brain and skull, and if bleeding is uncontrolled, it can cause major pressure problems for the brain and skull. Subarachnoid hemorrhages make up about 7% of all strokes. But they are the most deadly, with more than a 50% fatality rate. Research also shows that about half the survivors have permanent disabilities. Subarachnoid hemorrhages have such a high rate of fatality rate, it is believed, because of a common complication that can occur days to weeks after the bleeding. This complication is called cerebral vasospasm, which is the constriction of blood vessels within the brain. The constriction causes a lack of blood flow to portions of the brain, resulting in severe damage or death in regions of the brain.

Traditionally, treatment to prevent this condition is three weeks of strict bed rest after SAH and the Triple “H” Therapy. This uses hypervolemia that

just means increasing the blood volume to the brain by injecting saline into the veins, hemodilution that increasing plasma into the blood, decreasing the number of red blood cells and hypertension to increase the blood pressure. The idea behind this treatment is that if there is an increase in circulation, then the vessels are less likely to constrict. However, Dr. Eric DeShais, a Vascular Neurosurgeon at SUNY Upstate, is conducting patient clinical trials that suggest that if patients ambulate, or get up and walk around, right after surgery, this actually decreases the possibility of a vasospasm. The theory is that because there is less blood flow in the brain, there are decreased chances of complications, such as a thrombosis or blood clot.

Still, there is currently no medically proven treatment for cerebral vasospasm. This means that the condition cannot be prevented or managed to decrease severity and duration. The goal of my research is to create a reliable, reproducible animal model that suffers SAH and cerebral vasospasm. With this model, we characterized both histological and molecular vasospasm and tested to several avenues of neuroprotective strategies.

Our model organism was a Sprague-Dawley male rat because they are inexpensive, easy to work with and have similar physiological responses in SAH as humans. Technically, primates would be the best animal to stimulate a human delayed vasospasm because they have the most similar brain size and skull to humans. But the cost of an individual animal would be significant, and animal rights issues would make working with these animals virtually impossible.

Our surgery consists of two injections of blood into the outer portion of the brain 24 hours apart. The autologous blood was taken through an incision within the tail. The rats were then sacrificed either 90 minutes after the second surgery or two days after the surgery to ensure that we had a side range of SAH and a large window to see the vasospasm. The brains were removed from the rats, sliced into small sections and mounted on slides.

My method of determining the histological characteristics of vasospasm was done my examining the basal artery, which is the major artery that supplies oxygen to the brain. If this artery was constricted, it was highly probable that the rest of the brain was not getting enough oxygen and a vasospasm was occurring. First, I compared light microscope pictures of blood-injected and saline-injected rats. There was a significant amount of corrugation and thickness in the artery wall of the blood-injected rats compared to the controls. This was confirmed by computer measurements using NIH Image J software.

Next, I did a series of staining for different proteins and antibiotics to look at the difference of expression between SAH and the control. The hematoxylin and eosin stain added contrast between muscle layers and nucleus of the cells. With this stain, we were able to see that the blood-injected animals had the nucleuses of the cells closer together, showing constriction, and the muscle layers were more densely packed. Stains for iNOS, eNOS, HSP-70/72 Antibodies were used to see what layer of tissue had enzymes activated in them. Each enzyme or protein was used to assess potential changes between

blood and saline injections. The iNOS stain was used to look for inflammation that occurred during stroke within the brain and could have caused vasospasm to occur. Inflammation is a primary reaction of the body under stress and, in this case, can actually case more problems because it causes vasoconstriction of the vessels. HSP70/72 is a protein that is generated in the outer layer of vessels in response to several types of stress, such as heat shock and specific chemicals. This should be present within the vessels when blood is injected. This was confirmed within my staining results, and pictures are included within the body of the Capstone. Lastly, Masson's trichrome and Verhoeff stain was a three-color stain that stained the different layers of muscle, collagen and fibers separately. We were able to determine that there was a major increase in fibers in blood-injected vessels.

To look at the tissue molecular differences, I used extensive Polymerase Chain Reaction (PCR) assays, which involves amplification of short segments DNA from double-stranded DNA corresponding to specific mRNAs that are markers of cerebral vasospasm. This is a quantitative technique to reliably measure differences in such mRNAs causing more molecular data to be observed about blood injection versus saline. As was expected, there was a significant increase in the mRNAs found in the blood-injected rats compared to those injected with saline. This makes sense because these rats were under significant stress, causing their bodies to make more proteins to try and alleviate the pain.

Finally, to determine the exact molecular differences between the blood injection, I used a PCR Array specially for heat shock proteins. Each array contains 84 HSP genes and several housekeeping genes as controls. With my funds from SU Honors, I purchased the reagents needed to prepare the mRNA for analysis. PCR arrays are expensive, but they offer the opportunity to examine more than 80 mRNAs at once, as opposed to studying one molecule at a time. This is important because it provides a more complete picture of many molecules that change with cerebral vasospasm.

Results showed that a large decrease—six-fold—between the 90-minute blood-injected animals and the two-day blood-injected animals. This could lead to an increase in expression of heat-shock proteins early within SAH and decrease quickly over time. However, due to the time constraint, this research portion is in its early stages and requires more development.

The final goal of my project was to look at neuroprotective strategies of exercise pre-conditioning and injection of Stannous Choride. Stannous chloride has shown to be protective in inducing protective proteins during times of stress. Our data showed that 3 of 6 subjects showed reduced evidence of vasospasm when injected with blood and SnCl2, compared to saline controls. Exercise pre-conditioning could be a translation of Dr. DeShais clinical work—that if a patient is up and walking around, then vasospasm will be decreased in severity and duration. However, these results are inconclusive and need to be run under more stringent conditions.

There is still a considerable amount of research that needs to be conducted in this field, but my project has started to characterize SAH and vasospasm both histologically and molecularly. With these promising new results, I hope that in the near future, there will be a safe and effective treatment for patients suffering from SAH and vasospasms.